Differential regulation of Cdc2 and Aurora-A in *Xenopus* oocytes: a crucial role of phosphatase 2A

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Summary

The success of cell division relies on the activation of its master regulator Cdc2-cyclin B, and many other kinases controlling cellular organization, such as Aurora-A. Most of these kinase activities are regulated by phosphorylation. Despite numerous studies showing that okadaic acid-sensitive phosphatases regulate both Cdc2 and Aurora-A activation, their identity has not yet been established in *Xenopus* oocytes and the importance of their regulation has not been evaluated. Using an oocyte cell-free system, we demonstrate that PP2A depletion is sufficient to lead to Cdc2 activation, whereas Aurora-A activation depends on Cdc2 activity. The activity level of PP1 does not affect Cdc2 kinase activation promoted by PP2A removal. PP1 inhibition is also not sufficient to lead to Aurora-A activation in the absence of active Cdc2. We therefore conclude that in *Xenopus* oocytes, PP2A is the key phosphatase that negatively regulates Cdc2 activation. Once this negative regulator is removed, endogenous kinases are able to turn on the activator Cdc2 system without any additional stimulation. In contrast, Aurora-A activation is indirectly controlled by Cdc2 activity independently of either PP2A or PP1. This strongly suggests that in *Xenopus* oocytes, Aurora-A activation is mainly controlled by the specific stimulation of kinases under the control of Cdc2 and not by downregulation of phosphatase.

Key words: Aurora-A, Cdc2, PP1, PP2A, *Xenopus* oocyte

Introduction

Entry into both M phase of the first meiotic division of oocytes and mitosis of somatic cells rely upon the properly timed activation of the Cdc2-cyclin B kinase or MPF (M-phase promoting factor). Despite intensive study of Cdc2-cyclin B regulation, several well-documented molecules known to modulate Cdc2 activation, such as protein phosphatases 1 (PP1) and 2A (PP2A) are difficult to place in the Cdc2 regulatory pathway. Amphibian oocyte meiotic maturation is the pioneer model system to study MPF regulation. The full-grown *Xenopus* oocyte is physiologically arrested in meiotic prophase I and contains a maternal store of Cdc2 (or CDK1) associated with B2 and B5 cyclins (Hochegger et al., 2001). The cyclin B-Cdc2 complexes, which accumulate during oogenesis, are kept inactive by two inhibitory phosphorylations on Thr14 and Tyr15 of Cdc2. In *Xenopus*, meiotic maturation is induced by a steroid hormone, progesterone, which initiates a 3- to 5-hour transduction pathway resulting in the activation of the Cdc2-cyclin B complexes. This activation is achieved by Cdc2 dephosphorylation on Thr14 and Tyr15 inhibitory residues that is ensured by the protein phosphatase Cdc25 (for a review, see Karaiskou et al., 2001). A unique property of MPF in *Xenopus* oocytes is its ability to undergo autocatalytic activation in the absence of protein synthesis, and independently of the oocyte nucleus or GV (germinal vesicle), during the first meiotic division (Masui and Markert, 1971; Wasserman and Masui, 1974). This process is based on a positive feedback loop between Cdc2 and Cdc25 that allows the abrupt activation of MPF just before GVBD (germinal vesicle breakdown). It remains important to determine which molecular partners are involved in this autocatalytic process. Cdc2 activation parallels the hyperphosphorylation of Cdc25, which is required for its activation (Kumagai and Dunphy, 1992; Izumi et al., 1992). In vivo, two major kinases, Cdc2 itself and the *Xenopus* homologue of *Drosophila* Polo kinase, Plx1, phosphorylate and activate Cdc25 (Kumagai and Dunphy, 1996; Izumi and Maller, 1993). It has been shown that the initiation of the Cdc2/Cdc25 feedback loop requires two sets of phosphorylation reactions on Cdc25. The first depends on Cdc2 kinase and the second requires Plx1 activity (Karaiskou et al., 1999; Abrieu et al., 1998). The identity of the protein phosphatases that catalyze the dephosphorylation of Cdc25 is still uncertain. In vitro, both PP2A and PP1 dephosphorylate the hyperphosphorylated form of Cdc25 (Izumi et al., 1992). Furthermore, okadaic acid, a specific inhibitor of PP2A and PP1 phosphatases, induces MPF autoamplification (Felix et al., 1990; Goris et al., 1989). It has been proposed that Plx1 and an okadaic acid-sensitive phosphatase antagonistically regulate Cdc25 phosphorylation and activation, the phosphatase dephosphorylating the Plx1-phosphorylated residues of Cdc25 (Karaiskou et al., 1999). The okadaic acid-sensitive phosphatase has not been yet

unequivocally identified. The concentrations of okadaic acid required based on the different sensitivity of PP1 and PP2A is not informative because concentrated egg extract contains high levels of both phosphatases, and differences in sensitivity to okadaic acid of PP1 and PP2A are evident only in highly diluted extracts (Cohen, 1989; Lee et al., 1991). Moreover, Cdc25 activation is restrained through a PKA-dependent phosphorylation at Ser287 in prophase oocytes, a modification known to induce inhibition of Cdc25 through binding and sequestration by 14-3-3 (Kumagai et al., 1998). In response to progesterone, Cdc25 is dephosphorylated (Duckworth et al., 2002). Margolis and co-workers have reported that PP1 is responsible for dephosphorylating Ser287 of Cdc25 (Margolis et al., 2003). This result is consistent with a previous report indicating that injection of *Xenopus* oocytes with the PP1 inhibitor-1 (I-1) impedes Cdc2 activation and oocyte maturation (Huchon et al., 1981). Cdc25 activation would therefore depend on Ser287 dephosphorylation by PP1 but also on several activatory phosphorylations by Cdc2 and Plx1 at other sites that are negatively regulated by an unidentified okadaic acid-sensitive phosphatase.

Aurora-A and -B protein kinases are important factors in cell division control, Aurora-A being involved in spindle formation (Carmena and Earnshaw, 2003). Aurora-A plays various functions during *Xenopus* oocyte meiotic divisions, as the control of the first meiotic spindle (Castro et al., 2003) and more surprisingly the regulation of the polyadenylation of Mos mRNA (Mendez et al., 2000; Sarkissian et al., 2004). It has been shown that during oocyte maturation, active MPF is necessary and sufficient to induce indirectly Aurora-A phosphorylation and activation (Frank-Vaillant et al., 2000; Maton et al., 2003). Active Cdc2 could activate either an unknown kinase specific to Aurora-A or could allow Aurora-A to autophosphorylate and to autoactivate by recruiting an Aurora-A adapter or by inhibiting a specific phosphatase. Indeed, Aurora-A has two known activating partners, one localized in the centrosome, Ajuba (Hirota et al., 2003), and the other one in the mitotic spindle, TPX2 (Tsai et al., 2003; Eyers et al., 2003). Both of them stimulate autophosphorylation and autoactivation of Aurora-A on Thr295 in *Xenopus* (Thr288 in human Aurora-A) and protect the enzyme from inactivation by phosphatases (Tsai et al., 2003; Eyers et al., 2003). Recent studies reported that activation of Aurora-A kinase is negatively regulated by PP1. It was shown that the autoactivation of Aurora-A is regulated through a novel mechanism: Aurora-A autoactivation would be continuously antagonized by PP1 activity (Tsai et al., 2003). In the presence of regulatory proteins, such as TPX2, Ajuba or the PP1 inhibitor-2 (I-2), the dephosphorylation of active Aurora-A would be prevented (Hirota et al., 2003; Tsai et al., 2003; Eyers et al., 2003; Satinover et al., 2004). However, all these studies were conducted with bacterially expressed Aurora-A, which is activated by autophosphorylation. Whether this mechanism is operative in vivo remains to be shown, particularly during *Xenopus* oocyte meiotic maturation. In resting prophase oocytes, Aurora-A is kept inactive; it is activated during oocyte maturation following Cdc2 activation (Frank-Vaillant et al., 2000; Maton et al., 2003).

In this study, we asked two main questions. The first concerns the identification of the okadaic acid-sensitive phosphatases that negatively regulate Cdc2 and Aurora-A activation in *Xenopus* oocyte. The second relates to their regulatory role: does the activation of Cdc2 and Aurora-A depend solely on the inhibition of these phosphatases or does it require additional stimulation of regulatory kinases? To address these questions, we used microcystin beads that allow the generation of *Xenopus* oocyte extracts depleted in PP2A and retaining PP1.

Materials and Methods

Materials

Xenopus laevis adult females (Rennes, France) were bred and maintained under laboratory conditions. Reagents, unless otherwise specified, were from Sigma.

Purification of recombinant proteins

The 21 N-terminal amino acids of wild-type histone H3 fused with glutathione S-transferase (GST) were cloned into pGEX vector (Scrittori et al., 2001), expressed in bacteria and purified as described (Maton et al., 2003). Recombinant GST-p21^{cip1} was expressed in bacteria and purified as described (Frank-Vaillant et al., 1999). Rat DARPP-32 was expressed in bacteria and phosphorylated on Thr34 by PKA to a stoichiometry of one as described (Desdouits et al., 1995).

Prophase oocyte extracts

Fully grown *Xenopus* oocytes (prophase) or fully grown oocytes incubated overnight in 1 μM progesterone (metaphase II-arrested oocytes) were isolated and lysed at 4°C in four volumes of extraction buffer (EB) (80 mM β-glycerophosphate, pH 7.3, 20 mM EGTA, 15 mM $MgCl_2$, 1 mM DTT), supplemented with protease inhibitor mixture (Sigma, P8340). Lysates were centrifuged at 15,000 ***g*** at 4°C for 15 minutes. Supernatants (named 'oocyte extracts') were incubated at 30°C in the presence or absence of an ATP-regenerating system (10 mM creatine phosphate, 80 mg/ml creatine phosphokinase, 1 mM ATP, 1 mM $MgCl_2$) with or without 0.1 or 1 μM okadaic acid (ICN) or 0.1 or 1 μM microcystin-LR. Samples were collected at indicated times for Western blot analysis and kinase assays.

PP2A-depleted oocyte extracts

Extracts from 15 oocytes were incubated at 4°C for 1 hour with 10 μl microcystin-agarose beads (Upstate) or sepharose beads (Pharmacia) (v/v in EB). After centrifugation, the supernatant was collected and in some experiments, the incubation with beads was repeated once or twice. The bead pellets were resuspended in Laemmli buffer and analysed by western blotting. The supernatants were either collected for western blotting or processed for further incubation.

Western blotting

Samples equivalent to two oocytes were separated by a 12.5% SDS-PAGE Anderson system (Anderson et al., 1973) and transferred to nitrocellulose filters (Schleicher and Schuell) using a semi-dry blotting system (Millipore). The rabbit anti-*Xenopus* Aurora-A polyclonal antibody, the goat polyclonal antibody raised against *Xenopus* cyclin B2 and the guinea pig polyclonal antibodies directed against the 35 kDa catalytic subunit (C35) and the 65 kDa regulatory subunit (R65) of PP2A were described (Castro et al., 2002; De Smedt et al., 2000; Bosch et al., 1995) respectively. The mouse monoclonal antibody directed against the catalytic subunit of PP1 was purchased from Transduction Laboratories. The rabbit polyclonal antibody directed against Aurora-A phosphorylated on Thr295 (Thr288 in human Aurora-A), P-Aurora-A, was purchased from Cell Signaling.

The mouse monoclonal antibodies directed against DARPP-32 and the DARPP-32 phosphorylated on Thr34 (P-DARPP-32) were a kind gift of Paul Greengard (Rockefeller University, New York, NY) and have been described (Snyder et al., 1992). The primary antibodies were detected with appropriate horseradish peroxidase-conjugated second antibodies (Jackson ImmunoResearch Laboratories) and the western blot Chemoluminescence Renaissance kit from Perkin Elmer Life Sciences.

Kinase assays

To measure Cdc2 kinase activity, histone H1 assays were performed on $p13^{suc1}$ sepharose pull-down extracts (equivalent to three oocytes) as described (Jessus et al., 1991), in the presence of 1 μCi [γ-^{32}P]ATP (ICN), 100 μM ATP and 0.2 mg/ml histone H1 (Boehringer) in kinase buffer (50 mM Tris-HCl, pH 7.2, 15 mM $MgCl_2$, 5 mM EGTA, 1 mM DTT).

Histone H3 kinase activity of Aurora-A was assayed in immunoprecipitates (equivalent to ten oocytes), incubated for 30 minutes at 30°C in the presence of kinase buffer containing 3 μCi [γ-^{32}P]ATP (ICN), 50 μM ATP and 0.5 mg/ml recombinant H3-GST, as described (Maton et al., 2003). Kinase reactions were stopped by adding Laemmli buffer (Laemmli, 1970) and boiling. After electrophoresis and autoradiography, the bands corresponding to histone H1 and histone H3 peptide were excised and the associated radioactivity was measured in a Wallace 1409 scintillation counter.

Results

Okadaic acid or microcystin promote Cdc2 and Aurora-A activation

It is well established that addition of okadaic acid, a specific inhibitor of PP1 and PP2A (Bialojan and Takai, 1988), in prophase oocyte extracts leads to the activation of the Cdc2-cyclin B complex and Aurora-A in an ATP-dependent manner (Karaiskou et al., 1999; Maton et al., 2003). The minimal concentration that reproducibly induces Cdc2 activation corresponds to 10^{-7} M, a concentration allowing in vitro the inhibition of both PP1 and PP2A (Karaiskou et al., 1998). To determine whether PP1 or PP2A is involved in the negative regulation of Cdc2 and Aurora-A, we used another specific inhibitor of both phosphatases, microcystin (MacKintosh et al., 1990). We prepared low speed extracts (15,000 ***g***), known to retain all forms of PP2A and PP1, in contrast to high speed extracts (100,000 ***g***) that are deprived of the main active form of PP1 (Dumortier et al., 1987; Merlevede et al., 1984). Addition of either 10^{-6} M or 10^{-7} M microcystin or okadaic acid led to identical effects (Fig. 1). Cdc2 kinase was activated within one hour by both concentrations of okadaic acid and microcystin, as judged by the retardation of the electrophoretic mobility of cyclin B2, a well-established marker of Cdc2 activation (Fig. 1A). Cdc2 kinase activation was also directly ascertained by H1 kinase assay (Fig. 1B). Aurora-A phosphorylation was analysed by western blotting of extracts supplemented with either okadaic acid or microcystin. Both concentrations of either okadaic acid or microcystin induced Aurora-A phosphorylation, as judged by its electrophoretic shift (Fig. 1A), previously shown to correspond to the phosphorylation of the protein (Maton et al., 2003). Aurora-A was phosphorylated at least on the activatory residue Thr295, as revealed by an antibody recognizing specifically the Thr295-phosphorylated form of Aurora-A (Fig. 1A). As expected, Aurora-A phosphorylation was correlated with its kinase activity, assayed in Aurora-A immunoprecipitates (Fig. 1B). Therefore, 10^{-7} M or 10^{-6} M microcystin or okadaic acid is able to induce activation of Cdc2 and Aurora-A (Fig. 1). Knowing the elevated concentrations of PP1 and PP2A in a low-speed extract of *Xenopus* oocytes, 10^{-6} M or 10^{-7} M concentrations of microcystin or okadaic acid inhibit PP2A efficiently, and PP1 almost totally (Felix et al., 1990). Therefore, the specific phosphatase targeted by either okadaic acid or microcystin and responsible for the activation of both kinases cannot be identified by this experiment. To discriminate between the implications of either phosphatase, we took advantage of

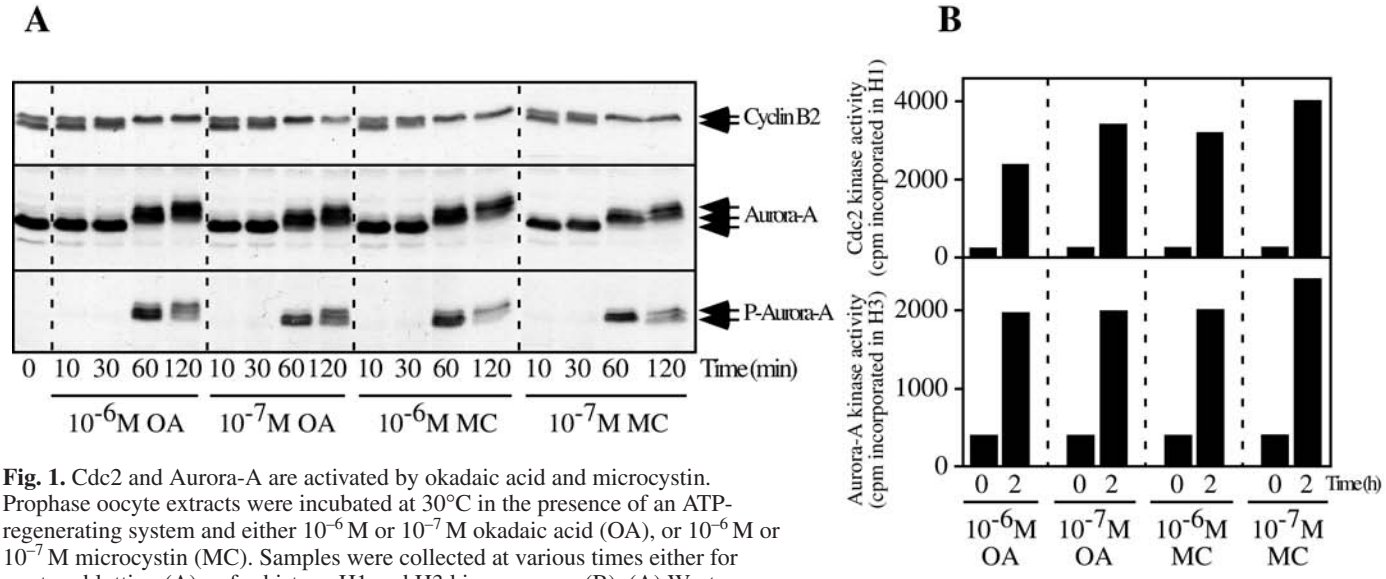

Fig. 1. Cdc2 and Aurora-A are activated by okadaic acid and microcystin. Prophase oocyte extracts were incubated at 30°C in the presence of an ATP-regenerating system and either 10^{-6} M or 10^{-7} M okadaic acid (OA), or 10^{-6} M or 10^{-7} M microcystin (MC). Samples were collected at various times either for western blotting (A) or for histone H1 and H3 kinase assays (B). (A) Western blots were analysed with the anti-Cyclin B2 antibody, the anti-Aurora-A antibody and the anti-P-Aurora-A antibody, as indicated. (B) Samples were pulled down on p13 beads and assayed for Cdc2 kinase activity using histone H1 as substrate, or immunoprecipitated with the anti-Aurora-A antibody and assayed for Aurora-A kinase activity using histone H3-peptide as substrate. Cdc2 and Aurora-A kinase activities are expressed in cpm incorporated in the substrate.

microcystin immobilized on agarose beads and found that these beads allow a total and rapid depletion of PP2A from *Xenopus* oocyte extracts.

Depletion of PP2A by microcystin beads

Prophase or metaphase II oocytes extracts were incubated in the presence of microcystin-conjugated agarose beads. After centrifugation to collect the beads, the supernatant was again incubated twice in the presence of the microcystin beads. All supernatants, corresponding to proteins that do not bind microcystin beads, and pellets corresponding to proteins retained on microcystin beads, were analysed by western blotting (Fig. 2A). The catalytic (C35) and regulatory (R65) subunits of PP2A were almost totally depleted from the extracts after one incubation with microcystin beads and totally removed from the extracts after a third passage on the beads (Fig. 2A); they were recovered in the pelleted microcystin beads (Fig. 2A). To assess the specificity of the binding, the same experiment was performed in the presence of 10^{-6} M okadaic acid, known to prevent microcystin binding. As expected, the presence of okadaic acid during the incubation with microcystin beads prevented the binding of PP2A on beads (Fig. 2A), showing the specificity of PP2A interaction with microcystin beads. Surprisingly, the amount of the catalytic subunit of PP1 in the extracts was not affected by three successive incubations in the presence of microcystin beads, in the presence or in the absence of okadaic acid (Fig. 2A). The amount of PP1 retained on the beads after the first incubation was low or even undetectable in some experiments (Fig. 2A). In any case, no decrease in PP1 could be visualized in the corresponding supernatants (Fig. 2A). The same procedure was performed with control beads that are not conjugated with microcystin (Fig. 2B). Control beads bound neither PP2A nor PP1 (Fig. 2B). We also ascertained that cyclin B2 and Aurora-A did not associate with microcystin beads or control beads (Fig. 2). Therefore, the use of microcystin beads allows the production of an oocyte extract that is depleted in PP2A but that retains PP1. We took advantage of the physical separation of both phosphatases to investigate their respective roles during Cdc2 and Aurora-A activation.

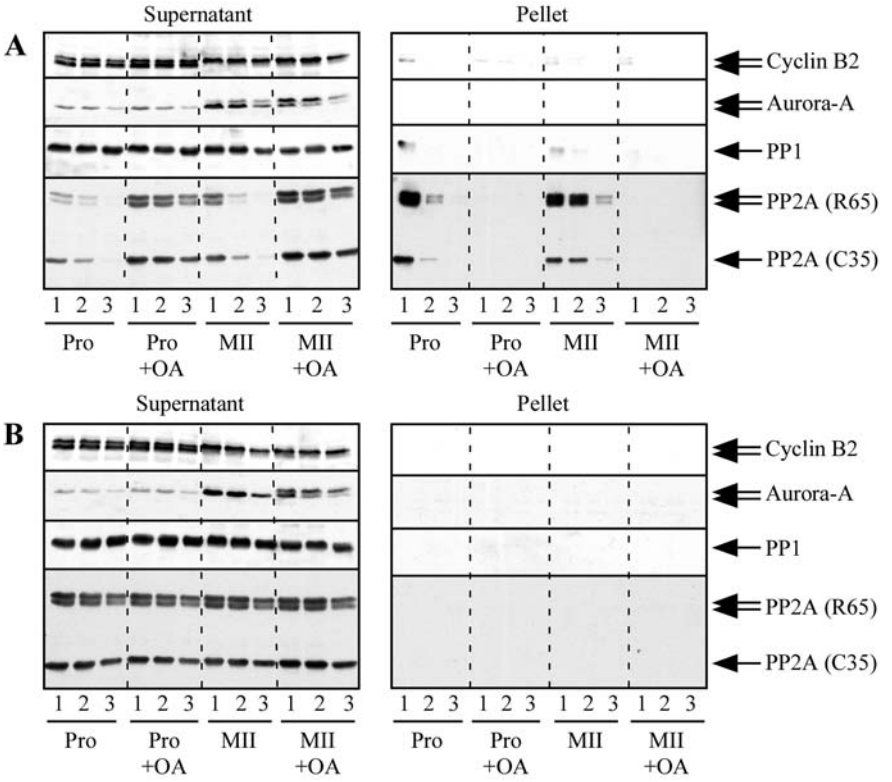

Fig. 2. Oocyte extracts are depleted in PP2A but retain PP1 after incubation with microcystin beads. Prophase oocytes (Pro) or Metaphase II-arrested oocytes (MII) were homogenized in the presence (+OA) or in the absence of 10^{-6} M okadaic acid. These extracts were incubated once (1), twice (2) or three times (3) for 1 hour at 4°C, either with microcystin-agarose beads (A) or with control beads (B). The supernatants (left panels) and the pellets (right panels) were analysed by western blotting with the anti-Cyclin B2 antibody, the anti-Aurora-A antibody, the anti-PP1 antibody and the anti-PP2A antibodies as indicated.

Removal of PP2A is sufficient to lead to Cdc2 and Aurora-A activation

Kinase activity of Cdc2 and Aurora-A was assayed in control extracts and in PP2A-depleted extracts after microcystin beads incubation (Fig. 3). In control extracts, activation of both kinases as well as phosphorylation of cyclin B2 and Aurora-A depend on the addition of okadaic acid together with an ATP-regenerating system (Fig. 3), as previously reported (Karaiskou et al., 1999; Maton et al., 2003; Karaiskou et al., 1998). In contrast, in PP2A-depleted extracts, all these events occurred spontaneously in the presence of an ATP-regenerating system, without requiring addition of okadaic acid: Cdc2 and Aurora-A kinases were activated (Fig. 3A) and cyclin B2 and Aurora-A underwent activatory phosphorylations (Fig. 3B). In some experiments, a partial kinase activation of Cdc2 and Aurora-A occurred spontaneously in PP2A-depleted extracts without any ATP addition, probably due to the presence of traces of endogenous ATP (Fig. 3). Addition of okadaic acid neither affected the time-course nor the level of the activation and phosphorylation of both kinases in PP2A-depleted extracts (Fig. 3). Therefore, removal of PP2A is sufficient to induce Cdc2 and Aurora-A activation, provided that ATP is present and despite the presence of PP1. This result suggests that a major negative control is exerted by PP2A on Cdc2 and Aurora-A in oocyte extracts. Once this negative regulator is removed, endogenous kinases are able to turn on the activatory system without any additional stimulation.

Activation of Aurora-A in the absence of PP2A depends on active Cdc2

We previously demonstrated that Aurora-A activation

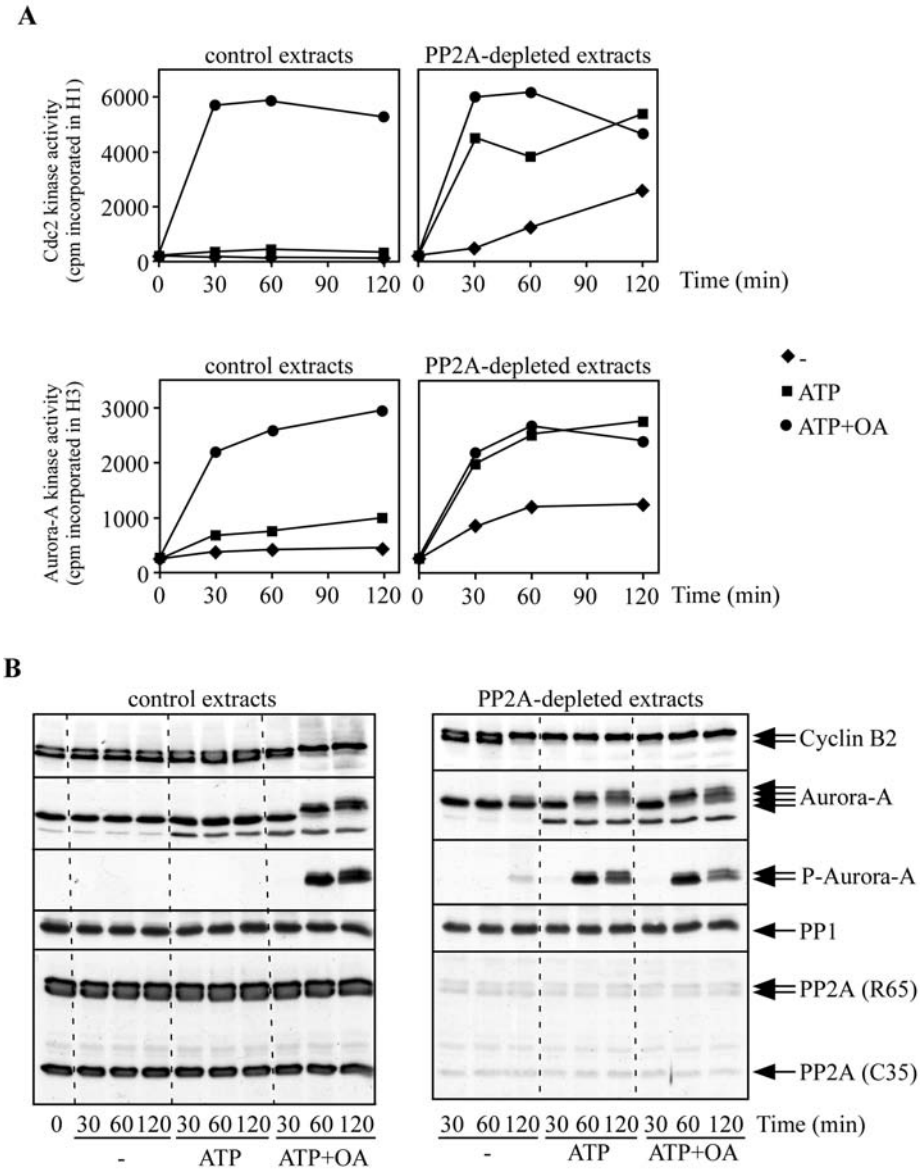

Fig. 3. Removal of PP2A is sufficient to lead to Cdc2 and Aurora-A activation. Prophase oocyte extracts were incubated in the absence or in the presence of microcystin-agarose beads. The extracts were then incubated at 30°C in the presence or in the absence (–) of an ATP-regenerating system (ATP) with or without okadaic acid (OA). Samples were collected at various times either for histone H1 and H3 kinase assays (A) or for western blotting (B). (A) Samples were pulled down on p13 beads and assayed for Cdc2 kinase activity using histone H1 as substrate, or immunoprecipitated with the anti-Aurora-A antibody and assayed for Aurora-A kinase activity using histone H3 peptide as substrate. Cdc2 and Aurora-A kinase activities are expressed in cpm incorporated in the substrate. (B) Western blots were analysed with the anti-Cyclin B2 antibody, the anti-Aurora-A antibody, the anti-P-Aurora-A antibody, the anti-PP1 antibody and the anti-PP2A antibodies as indicated.

depends on Cdc2 in oocyte extracts stimulated by okadaic acid, where both PP2A and PP1 are presumably inhibited (Maton et al., 2003). We then addressed the question whether Cdc2 is still required for Aurora-A activation in PP2A-depleted extracts that retain PP1. Control extracts or extracts previously incubated in the presence of either control beads or microcystin beads were supplemented with the CDK inhibitor, $p21^{Cip1}$, known to specifically inhibit Cdc2 activation in prophase oocytes (Frank-Vaillant et al., 1999). Cdc2 and Aurora-A activation were analysed by kinase assays and by estimating the phosphorylation level of cyclin B2 and Aurora-A by western blotting (Fig. 4). It should be noted that the recombinant GST-$p21^{Cip1}$ was recognized by the mixture of anti-PP2A antibodies, giving raise to a 67 kDa band migrating just above the regulatory PP2A subunit (R65) (Fig. 4). In non-treated extracts as well as in extracts incubated with control beads, Cdc2 and Aurora-A activation was induced by okadaic acid addition. As expected, $p21^{Cip1}$ prevented efficiently Cdc2 activation, and as a consequence, Aurora-A phosphorylation and activation in both extracts (Fig. 4A,B). In PP2A-depleted extracts, Cdc2 and Aurora-A activation occurred spontaneously in the presence of ATP and Cdc2 inhibition by $p21^{Cip1}$ prevented activation and phosphorylation of Aurora-A (Fig. 4C). Therefore, Aurora-A activation requires Cdc2 activity: removal of PP2A allows an endogenous kinase to induce Cdc2 activation, which in turn leads to the activation of Aurora-A.

Inhibition of PP1 does not modify Cdc2 and Aurora-A activation in the absence of PP2A

PP1 has been shown to regulate negatively recombinant Aurora-A autophosphorylation (Katayama et al., 2001). In contrast, PP1 was shown to regulate Cdc2 activation positively, through Cdc25 dephosphorylation on the inhibitory residue Thr287 (Margolis et al., 2003). As PP1 is present in PP2A-depleted extracts, it would then favour Cdc2 activation and impair Aurora-A activation. To further explore the role(s) of PP1 in the pathway leading to the activation of these kinases, its phosphatase activity was inhibited by adding the specific protein inhibitor, P-DARPP-32 (Walaas et al., 1983). This protein is not expressed in *Xenopus* oocytes. The inhibitory activity of DARPP-32 depends on its phosphorylation by PKA (Hemmings et al., 1984). Conversely, DARPP-32 is dephosphorylated and inactivated by PP2A and PP2B (Hemmings et al., 1990). We therefore used a recombinant phosphorylated form of DARPP32, P-DARPP32. In control extracts, Cdc2 and Aurora-A activation was induced by addition of okadaic acid, in the presence or in the absence of P-DARPP-32 (Figs 5, 6). Cdc2 and Aurora-A kinase activation (Fig. 5), as well as cyclin B2 and Aurora-A

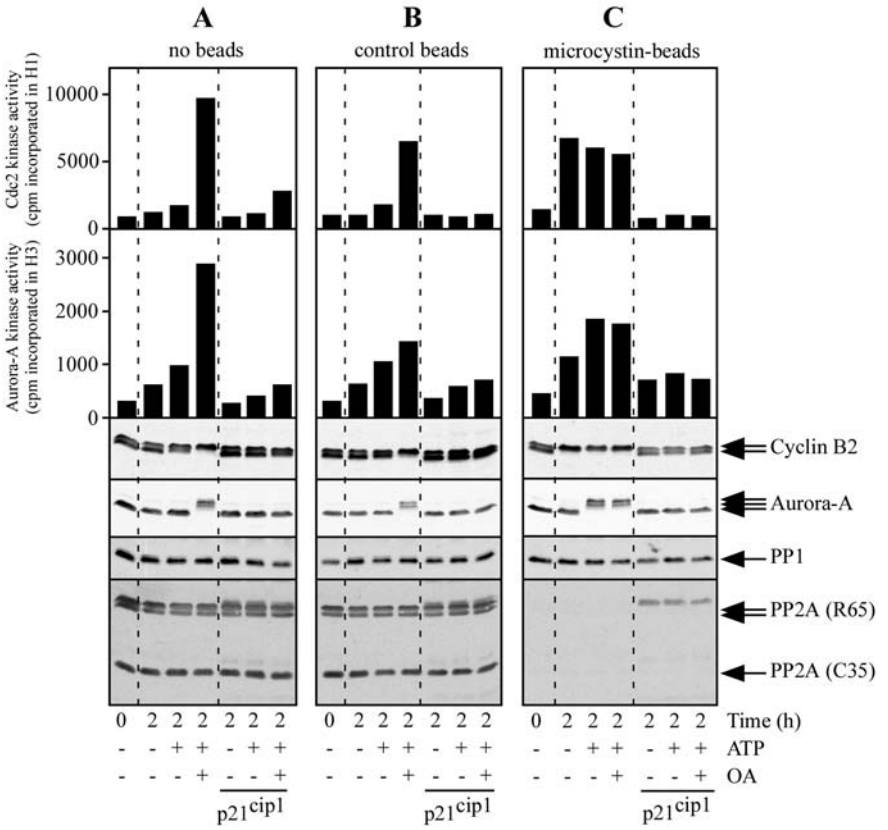

Fig. 4. Aurora-A activation induced by PP2A removal is dependent on Cdc2. Prophase extracts were incubated in the presence of 40 μg/ml of $p21^{Cip1}$ and then incubated (C) or not (A) with microcystin-agarose beads at 4°C. Control extracts were obtained by incubation with control beads at 4°C (B). After centrifugation, the supernatants were then incubated in the absence or in the presence of an ATP-regenerating system (ATP) with or without okadaic acid (OA) for 2 hours at 30°C. Samples were collected at indicated times either for histone H1 and H3 kinase assays or for western blotting. A pull-down assay on p13 beads was used to measure Cdc2 kinase activity using histone H1 as substrate. Immunoprecipitates were performed with the anti-Aurora-A antibody and assayed for Aurora-A kinase activity using histone H3-peptide as substrate. Cdc2 and Aurora-A kinase activities are expressed in cpm incorporated in the substrate. Western blots were probed with the anti-Cyclin B2 antibody, the anti-Aurora-A antibody, the anti-PP1 antibody, and the anti-PP2A antibodies as indicated.

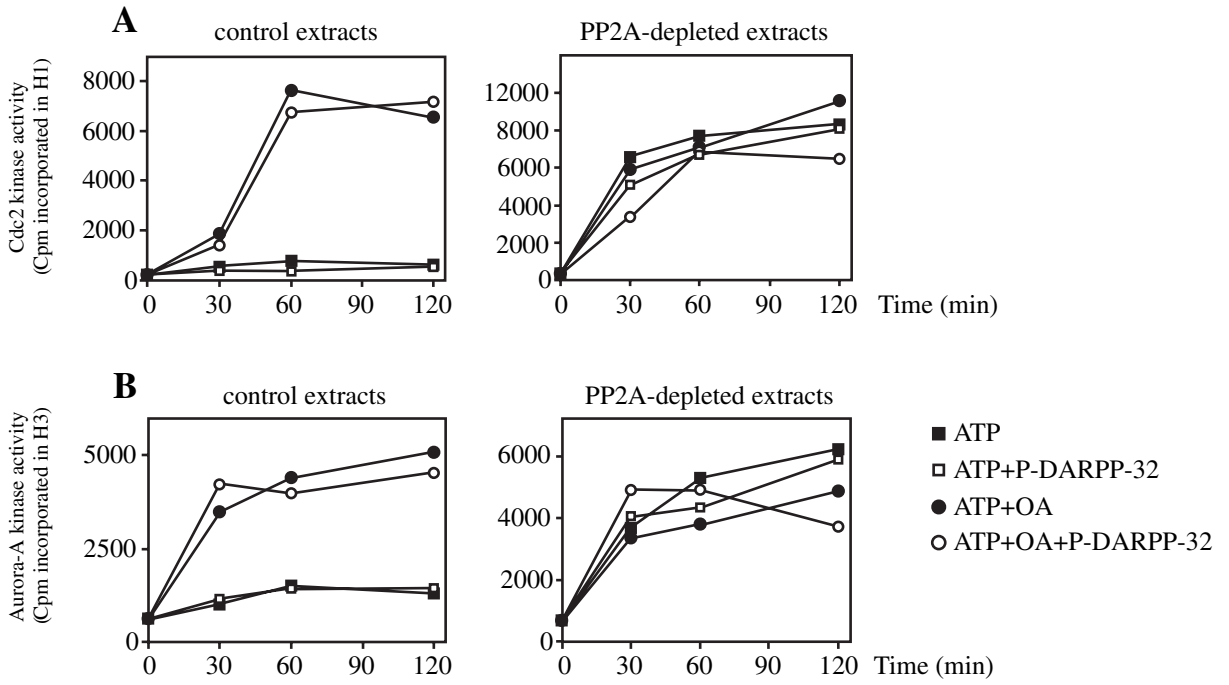

Fig. 5. Cdc2 and Aurora-A kinase activation is independent of PP1 inhibition. Prophase extracts were depleted (right panels) or not (left panels) in PP2A by incubation at 4°C with microcystin-agarose beads. The extracts were then incubated in the presence or absence of 500 nM P-DARPP-32 at 30°C with an ATP-regenerating system (ATP) plus or minus okadaic acid (OA). Samples were collected at indicated times for histone H1 (A) and histone H3 kinase assays (B). (A) Samples were pulled down on p13 beads and assayed for Cdc2 kinase activity using histone H1 as substrate. (B) Samples were immunoprecipitated with anti-Aurora-A antibody and assayed for Aurora-A kinase activity using histone H3-peptide as substrate. Cdc2 and Aurora-A kinase activities are expressed in cpm incorporated in the substrate.

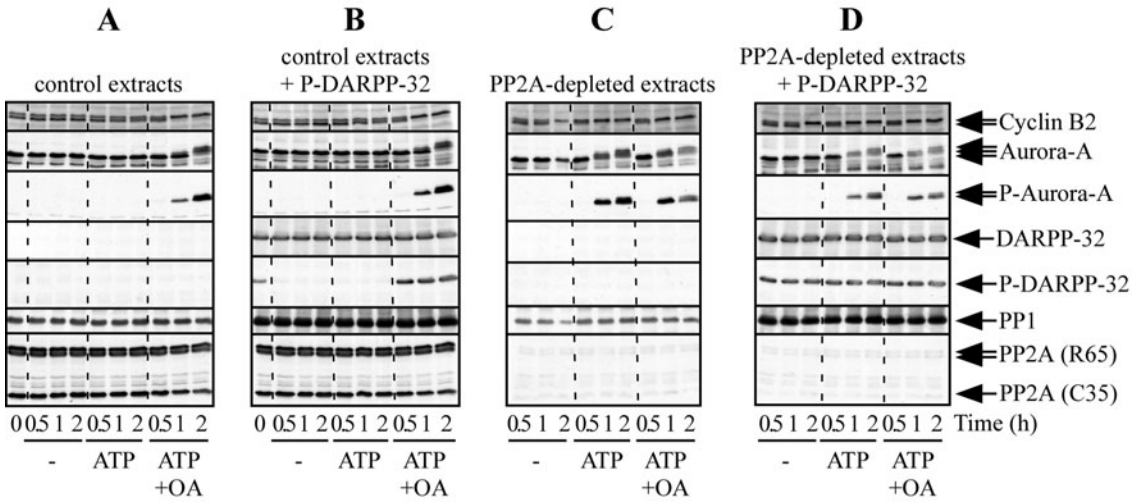

Fig. 6. Cyclin B2 and Aurora-A phosphorylation is independent of PP1 inhibition. The same extracts as in Fig. 5 were used. Prophase extracts were depleted (C and D) or not (A and B) in PP2A by incubation with microcystin-agarose beads. After centrifugation, the extracts were incubated in the presence (B and D) or absence (A and C) of 500 nM P-DARPP-32 at 30°C in the absence (–) or in the presence of an ATP-regenerating system (ATP) plus or minus okadaic acid (OA). Samples were collected at indicated times and analysed by western blotting with the anti-Cyclin B2 antibody, the anti-Aurora-A antibody, the anti-P-Aurora-A antibody, the DARPP-32 antibody, the anti-P-DARPP-32 antibody, the PP1 antibody and the PP2A antibodies, as indicated.

phosphorylation (Fig. 6A,B) were not affected by the presence of P-DARPP-32.

The presence of exogenously added DARPP-32, as well as its phosphorylation level, were analysed by western blotting using specific antibodies, recognizing either all the forms of DARPP-32 or its phosphorylated form. We observed that in control extracts, P-DARPP-32 was dephosphorylated within 30 minutes in the absence of okadaic acid, whereas the phosphorylated form was stable over 2 hours in the presence of okadaic acid (Fig. 6B). This result indicates that active PP2A present in crude extracts dephosphorylates and therefore inactivates DARPP-32. In the presence of okadaic acid, DARPP-32 phosphorylation remained stable, owing to PP2A inhibition, and thus capable of inhibiting PP1. The okadaic acid concentration used in our experiments is probably not sufficient to fully abolish PP1 activity in crude extracts (Goris et al., 1989; Karaiskou et al., 1998). Our results therefore show that full inhibition of PP1 by P-DARPP-32 in okadaic acid-treated extracts does not affect Cdc2 and Aurora-A activation (Fig. 5, Fig. 6A,B).

We then analysed the effect of PP1 inhibition by P-DARPP32 in PP2A-depleted extracts. As expected, DARPP-32 phosphorylation was stable in such extracts, owing to the absence of PP2A, and the inhibitor was therefore active towards PP1 all over the incubation period (Fig. 6D). In PP2A-depleted extracts, Cdc2 and Aurora-A activation occurred spontaneously within 1 hour in the presence of ATP (Fig. 5, Fig. 6C). Inhibition of PP1 by P-DARPP-32 did not modify the time-course, the activation level and the phosphorylation level of Cdc2, cyclin B2 and Aurora-A (Fig. 5, Fig. 6C,D). Under such conditions, it is however impossible to know whether PP1 inhibition is sufficient to induce Aurora-A activation as Cdc2 is activated by PP2A removal and is able to trigger Aurora-A activation indirectly.

To investigate whether PP1 inhibition is sufficient to promote Aurora-A activation independently of Cdc2 and PP2A, extracts were depleted in PP2A in the presence of $p21^{Cip1}$ (note again in Fig. 7 that $p21^{Cip1}$ was non-specifically recognized by PP2A antibodies in western blots, giving rise to a 67 kDa $p21^{Cip1}$ band and a 36 kDa cleavage product, probably GST). Under such conditions, extracts are devoid of PP2A but retain PP1, and Cdc2 activation is prevented by the $p21^{Cip1}$ inhibitor (Fig. 7). P-DARPP-32 was added in such extracts. Inhibition of PP1 by P-DARPP-32 is unable to trigger phosphorylation on Thr295 and activation of Aurora-A, even in the presence of ATP (Fig. 7). Then, it can be concluded that the regulation of Aurora-A activation is driven by Cdc2 activity and is independent of PP1.

Discussion

It has previously been unclear which okadaic acid-sensitive phosphatase negatively regulates Cdc2 activation and the burst of numerous downstream kinases, such as Aurora-A, that occur during *Xenopus* oocyte meiotic maturation. In this report, we show that it is possible to use microcystin beads to eliminate the main trimeric form of PP2A. Under our experimental conditions, most of the PP1 catalytic subunit remains in the supernatant after removal of the pellet of microcystin beads. The observation that microcystin beads do not bind PP1 catalytic subunit in the *Xenopus* extracts suggest that PP1 is associated with regulatory subunits that prevent its binding to microcystin.

More than a decade ago, it was proposed that PP2A, a major Ser/Thr phosphatase present in the *Xenopus* oocyte, is a negative regulator of Cdc2 activation (Hermann et al., 1988; Lee et al., 1994). Our results based on the use of PP2A-depleted extracts unequivocally show that PP2A corresponds to the okadaic acid-sensitive phosphatase that antagonizes the autoamplification of Cdc2. The simple addition of ATP to PP2A-depleted extracts allows Cdc2 activation. Inhibition of PP1 by the specific inhibitor P-DARPP-32, does not affect

Cdc2 activation. This result means that the activation of latent kinase(s) antagonized by PP2A is sufficient for the activation of Cdc25C, the phosphatase necessary for Cdc2 autoamplification. It is well established that activity of Cdc25 in *Xenopus* oocytes is positively regulated by Cdc2 kinase and Plx1 (Kumagai and Dunphy, 1996; Karaiskou et al., 1999; Abrieu et al., 1998). Plx1 activity is under the control of Cdc2 (Karaiskou et al., 1999; Abrieu et al., 1998; Erikson et al., 2004). It can therefore be concluded that in prophase-arrested oocytes, PP2A permanently antagonizes the activity of these kinases. All these results would neatly explain why a threshold amount of active Cdc2 is sufficient to initiate the autoamplification loop: the presence of a starter amount of active Cdc2 would activate Plx1; both Cdc2 and Plx1 would then overcome the action of PP2A and prevent it dephosphorylating Cdc25. Therefore, the autoamplification loop can be initiated in two ways: PP2A inhibition and/or an increase in Cdc2 activity above a threshold level. The pathway selected by progesterone, the physiological inducer of M-phase entry in *Xenopus* oocytes, remains to be determined. In any case, our experimental approach definitively shows that PP1 is not the okadaic acid-sensitive phosphatase preventing Cdc2 autoamplification.

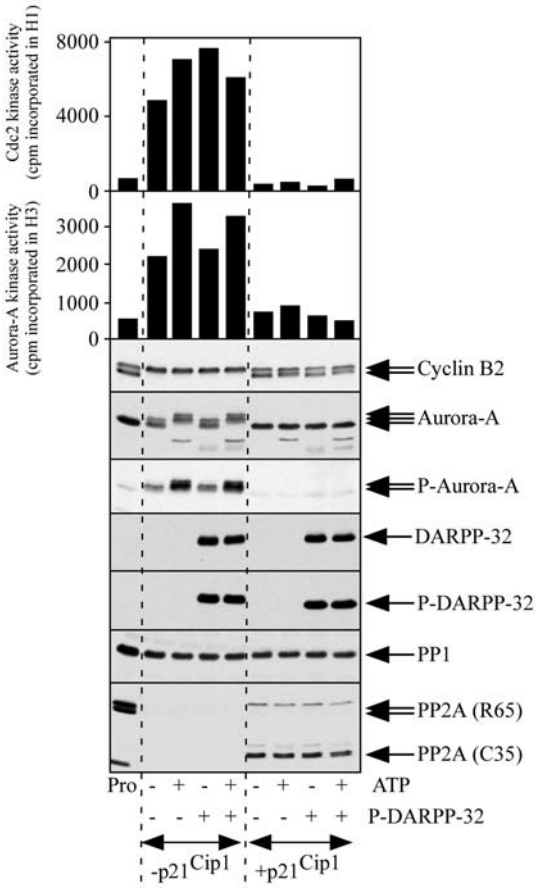

Fig. 7. PP1 inhibition is not sufficient to trigger Aurora-A phosphorylation and activation. Prophase extracts (Pro) were depleted in PP2A by incubation with microcystin-agarose beads in the presence or in the absence of $p21^{Cip1}$. After centrifugation, the extracts were incubated for 2 hours in the presence or in the absence of 500 nM P-DARPP-32 at 30°C plus or minus an ATP-regenerating system (ATP). Samples were collected for kinase assays and western blots. A pull-down assay on p13 beads was used to measure Cdc2 kinase activity using histone H1 as substrate. Immunoprecipitates were performed with anti-Aurora-A antibody and assayed for Aurora-A kinase activity using histone H3-peptide as substrate. Cdc2 and Aurora-A kinase activities are expressed in cpm incorporated in the substrate. Western blots were probed with the anti-Cyclin B2 antibody, the anti-Aurora-A antibody, the anti-P-Aurora-A antibody, the DARPP-32 antibody, the anti-P-DARPP-32 antibody, the PP1 antibody and the PP2A antibodies, as indicated.

As described in the introduction, the phosphorylation and the kinase activation of Aurora-A in *Xenopus* oocytes requires the previous activation of Cdc2 kinase both in vivo and in vitro (Frank-Vaillant et al., 2000; Maton et al., 2003). The link between Cdc2 and Aurora-A remains unknown but it does not involve a direct phosphorylation of Aurora-A by Cdc2 (Maton et al., 2003). An interesting model for Aurora-A has recently been proposed (Tsai et al., 2003; Eyers et al., 2003); it indicates that *Xenopus* Aurora-A is able to autoactivate by phosphorylating its Thr295 residue (Thr288 in humans). Aurora-A contains consensus sequences involved in PP1 catalytic subunit binding and autophosphorylation of a recombinant form of Aurora-A is antagonized by PP1 in vitro (Katayama et al., 2001). Interaction with new regulators, such as TPX2, Ajuba or the PP1 inhibitor-2 (I-2) prevents the antagonistic action of PP1 and leads to Aurora-A activation (Hirota et al., 2003; Eyers et al., 2003; Satinover et al., 2004). Then Aurora-A activation could simply rely on PP1 inhibition. Therefore it was important to determine whether this mechanism involving PP1 is operative in vivo and/or in vitro in oocyte extracts and whether it is independent of Cdc2 activity.

Our results show that removal of PP2A from oocyte extracts by microcystin beads is sufficient to lead to Aurora-A activation, despite the presence of PP1. Removal of PP2A and inhibition of PP1 by the DARPP-32 inhibitor does not modify Aurora-A activation. Under these conditions, we have shown that the direct consequence of PP2A removal is Cdc2 activation, and that active Cdc2 is necessary for Aurora-A activation. Cdc2 activity is absolutely required for Aurora-A activation in PP2A-depleted extracts, whether PP1 is active or inhibited by P-DARPP-32. Moreover, our results unambiguously show that the inhibition of PP1 by DARPP-32 is not sufficient to induce phosphorylation and activation of Aurora-A in the absence of Cdc2 activity.

In *Xenopus* oocytes, Aurora-A activation depends on activation of a stimulatory pathway initiated by Cdc2, which does not require PP1 inhibition. Whether this pathway involves proteins already known to play a role in Aurora-A activation in mitotic cells, such as TPX2, Ajuba or I-2, and whether it implicates Aurora-A autophosphorylation or the recruitment of new protein kinases, remain to be explored. Our results do not support the view that the regulation of Aurora-A activity in resting prophase oocytes is regulated by a permanent autophosphorylation/dephosphorylation turnover.

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